Original Article

Degradation of Atrazine by Soil Bacteria in the Stationary Phase

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Two atrazine-degrading bacteria, RK014 from Anjo (Japanese) soil and RK016 from Pak Chong (Thai) soil, were isolated. The isolate RK014 was an unidentified, Gram negative and non spore-forming rod, while RK016 was classified into *Bacillus* sp. The study on the degradation of atrazine in tenfold diluted nutrient broth containing 10 mg/l of atrazine revealed that atrazine was degraded mainly at stationary phase in both bacterial cultures. Higher than 10^8 – 10^9 cfu/ml of initial cell density was required for rapid degradation. The degradation rate of atrazine was higher at pH 8 than pH 5 in both cultures with the high density of inoculum. Deethylatrazine was a major metabolite by both isolates in the cultures. It is suggested that these characteristics of atrazine-degrading bacteria caused the slow microbial degradation of atrazine in soils in spite of ubiquitous presence of degrading microorganisms at 10^5 MPN/g-soil.

INTRODUCTION

The ubiquity of degraders among Thai and Japanese soils was suggested by the findings of Korpraditskul *et al.*¹⁾ that the population of atrazine-degrading microorganisms was higher than 10^5 MPN/g-soil in the two soils examined. The microbial degradation in the soils appeared to take a distinctive role in atrazine degradation after 20 days of incubation, though in a minor contribution. The low and slow appearance of activity in the microbial degradation of atrazine in the soils was discordant to the relatively high population of atrazine degraders. It was reported that atrazine was rapidly degraded by some soil fungi and that 73% of atrazine was deethylated within 32 days.²⁾ There was also the evidence that soil microorganisms could degrade *s*-triazine herbicides in the pure cul-

tures.³⁻⁶⁾

In order to elucidate this contradictory phenomenon, the atrazine-degrading bacteria were isolated from the soils and the characteristics in atrazine degradation was examined in this study.

MATERIALS AND METHODS

1. Standard Chemicals

Reference standards used were atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine, 99.3% of purity), deethylatrazine (DEA, 4-amino-2-chloro-6-isopropylamino-1,3,5-triazine, 98.2% of purity) and deisopropylatrazine (DIA, 6-amino-2-chloro-4-ethylamino-1,3,5-triazine, 98.0% of purity). DEA was contained in standard atrazine at 0.013% and DIA was less than 0.001%. All reference standards were gifted from Ciba-Geigy (Japan) Limited.

2. Soil

Anjo (Japanese) and Pak Chong (Thai) soils were used to isolate atrazine-degrading microorganisms. Soil preparations and properties

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were reported previously.^{1,7)}

3. Isolation of Atrazine-Degrading Microorganisms

Atrazine-degrading microorganisms were isolated from the cultures in dilution series with a Most Probable Number method reported by Korpraditskul *et al.*¹⁾ The highest-diluted cultures that showed the decrease of atrazine or those which exhibited peaks of metabolites on the gas chromatograms were used for the isolation by a spread-plate method. The agar medium was made by tenfold diluted nutrient broth (Eiken, Tokyo, Japan) and 1.5% agar (Nakarai Chemical, Tokyo, Japan). Colonies formed were further transferred repeatedly on the medium until each pure culture was obtained. The dominant microbes were judged based on the morphology of colonies after the observation by a microscopy with a low-power magnification. Each purified colony was cultured in tenfold diluted nutrient broth (1/10NB) for 24 hr (pH 6.8, 30°C). Cells were centrifuged at $10,000 \times g$ for 10 min and washed with a physiological saline. The washed cell suspension was inoculated to give higher than 10^8 cfu/ml of density for Gram-positive bacteria and higher than 10^9 cfu/ml of density for Gram-negative ones into 10 ml of fresh 1/10NB containing 10 mg/l of atrazine. The control without inoculation was prepared in parallel. Atrazine was added as 100 μ l of acetone solution after sterilization passing through a membrane filter with 0.2 μ m of pore size. After appropriate intervals of incubation, the amount of atrazine remaining in the culture was determined as described below. The degradation of atrazine was evaluated by the percentage decrease based on the amount of atrazine remaining in control.

4. Taxonomic Characterization of Isolates

Some taxonomic characteristics of isolates were tested for identification. Catalase production was determined with 3% H_2O_2 .⁸⁾ Oxidase test was with a tetramethyl-*p*-phenylene-diamine dihydrochloride test paper (oxidase test paper,[®] Eiken, Tokyo, Japan).⁹⁾ OF-test was carried out using a commercial OF test media by supplier's protocol (Nissui,

Tokyo, Japan).

5. Growth Stage and Atrazine Degradation

The 1/10NB containing 10 mg/l of atrazine (pH 6.8) was inoculated with one loopful of the 24 hr-old culture of the isolate RK014 or RK016, which gave 10^8 to 10^4 cfu/ml of density. Under shaking condition at 30°C, the growth was monitored by means of OD_{660} and the concentration of atrazine remaining in the medium was measured by gas chromatography. The control without inoculation was also prepared in parallel. The degradation was evaluated by the comparison of remaining atrazine.

6. Effect of pH on Atrazine Degradation

The isolate RK016 was cultured in 1/10NB. At an early stationary phase of growth, the cells were collected, washed with sterile water and inoculated into 10 ml of a basal salt medium, which consisted of 1.6 g of K_2HPO_4 , 0.4 g of KH_2PO_4 , 0.3 g of NH_4NO_3 , 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl and 0.02 g of $CaCl_2$ in 1 l of distilled water. The initial cell density was 10^8 cfu/ml in the medium. Before the inoculation, pH of the medium was adjusted to 4 and 5 with 100 mM citrate-200 mM phosphate buffer, and at pH 6, 7 and 8 with 100 mM phosphate buffer. Atrazine was added to the culture as described above. The initial concentration of atrazine was 10 mg/l. The control was prepared in the same manner after killing cells by autoclaving. After 30 days of incubation, the remaining amount of atrazine was determined as described below and the degradation percentage was calculated by the remaining amount in the control culture. The viable cells after 30 days of incubation were enumerated using an agar medium that consisted of the same basal salts as above and 0.1 g/l of glucose instead of atrazine as carbon source.

7. Atrazine and N-Dealkylated Metabolites Determination

After incubation, to determine the concentration of atrazine and its *N*-dealkylated metabolites in the culture, 2 ml of the culture suspension was transferred to a screw-capped tube, mixed with 1 ml of acetone and 2 ml of

10% NaCl, and then extracted with 3 ml of diethyl ether by shaking mechanically for 30 min. The extract was directly analyzed by gas chromatography since there was no interfering peak. The operating conditions were described in the previous report.¹⁾ The recoveries of atrazine, deethylatrazine and deisopropylatrazine were $93 \pm 8\%$, $86 \pm 9\%$ and $78 \pm 8\%$, respectively.

RESULTS AND DISCUSSION

1. Isolation of Atrazine Degraders

In the previous paper,¹⁾ atrazine-degrading microorganisms were enumerated by a Most Probable Number method. From the positive cultures in the experiment, which showed the decrease in atrazine amount, microorganisms were isolated by a dilution plate technique. The dominant bacteria judged by morphology of colonies, 5 isolates from Pak Chong soil and 8 isolates from Anjo soil, were obtained. Atrazine-degrading ability was examined for each isolate using a large inoculum size giving higher than 10^8 to 10^9 cfu/ml of density. The isolate RK014 from Anjo soil degraded 25.5% of atrazine after 9 days of incubation and the isolate RK016 from Pak Chong soil degraded 35.2% of atrazine.

To isolate atrazine-catabolizing bacteria, some kinds of mineral media containing atrazine as the sole carbon and/or nitrogen source were also used. However, no degrader was obtained. This suggests that dominant soil microorganisms do not degrade atrazine catabolically but incidentally.

Figure 1 shows that both isolates were rod-

Table 1 Taxonomic characteristics of the atrazine-degrading bacteria isolated.

	Isolates	
	RK014	RK016
Gram reaction	—	+
Shape	Rod	Rod
Spore	—	Endospore
Mobility	+	+
Catalase	+	+
Oxidase	+	+
OF-test	—	Oxidative

shaped bacteria. Some taxonomic characteristics of the two isolates are shown in Table 1. Isolate RK014 was an unidentified, non spore-forming, Gram-negative bacterium. The isolate RK016 carrying endospore was identified as *Bacillus* sp.⁸⁾ Bacteria classified into the genus *Bacillus* sp. have been reported as the major bacteria in Pak Chong soil.⁹⁾

2. Growth Stage and Atrazine Degradation

Figure 2 shows that atrazine was degraded by both bacteria RK014 and RK016 in the stationary phase. Cell proliferation monitored by OD₆₆₀ showed that the growth of RK016 was slightly faster than RK014. Isolates RK014 and RK016 started the exponential growth after 14 and 10 hr of lag periods, respectively. After one day of incubation, the OD₆₆₀ values became the highest that corresponded to 1.8×10^8 cfu/ml for RK014 and 2.4×10^7 cfu/ml for RK016. Then, the OD₆₆₀ values decreased sharply and remained con-

Fig. 1 Morphological shape of the isolates RK016 (A) and RK014 (B) after 24 hr of incubation in tenfold diluted nutrient broth.

stant for a long time. In this stationary phase, both bacteria kept 10^6 cfu/ml of population. The degradation of atrazine occurred after 8 days of incubation in the culture of RK014 and after 10 days in the culture of RK016. The change of pH mainly occurred during the exponential growth phase and the pH value became constant at 8 in the stationary phase. Chemical degradation of atrazine does not occur at pH 8, which was shown by less than 3% decrease in atrazine concentration after 30 days in phosphate buffer at pH 8. Thus, atrazine was degraded through biological activity. This is the first evidence that atrazine is degraded by microorganisms at stationary phase.

In the past, many microorganisms capable

Fig. 2 Changes of pH, optical density at 660 nm and the atrazine concentration in the cultures of atrazine-degrading bacteria RK014 (●) and RK016 (○).

Atrazine concentration in control culture is represented by □. Bars represent the standard deviation of triplicated experiment.

of degrading s-triazine herbicides including atrazine have been isolated.^{2-6,10,11)} A slow and continuous rate of degradation has often been observed.¹⁰⁾ An incidental or nonpreferential involvement of s-triazine herbicides in microbial metabolism has been suggested as a degrading mechanism.¹⁰⁾ However, there has been no report on relation between the degradation and the physiology of microorganisms, *i.e.* the growth stage.

3. Effect of pH on Atrazine Degradation

The washed cells of RK016 were inoculated into basal salt medium and incubated for 30 days. The results shown in Fig. 3 revealed the highest rate of degradation occurred at pH 8. The same tendency was observed in the case of RK014 as shown in Table 2. The higher degradation rate at higher pH is consistent with the previous findings¹⁾ that the contribution of microbial degradation to total atrazine degradation was higher in soils at pH 7 and 8 than at pH 4, 5 and 6.

The initial cell density of RK016 inoculated was 10^8 cfu/ml. Viable population decreased to about 10^6 cfu/ml during incubation irrespective of pH values. This agreed with the

Fig. 3 Degradation of atrazine by isolate RK016 in basal salts medium after 30 days of incubation.

Adjustment of pH was carried out using citrate-phosphate buffer for pH 4, 5 and 6 and phosphate buffer for pH 7 and 8. The percentage of atrazine degradation (□) was calculated based on the remaining amount of atrazine in the control culture to cancel the effect of chemical degradation. The initial density of RK016 was 10^8 cfu/ml. The number of viable cells (▲) was counted after the incubation in the same medium with glucose instead of atrazine. Bars represent the standard deviation of triplicated experiment.

Table 2 The concentrations of atrazine and *N*-dealkylated metabolites detected in the cultures of RK014 and RK016 after 3 months of incubation.^{a)}

	pH 8			pH 5		
	ATZ ^{b)} (mg/l)	DEA ^{b)} (μg/l)	DIA ^{b)} (μg/l)	ATZ (mg/l)	DEA (μg/l)	DIA (μg/l)
Control	11.1	115.2	9.1	7.1	50.0	12.0
RK014	9.9	658.7	76.9	7.9	320.0	23.0
RK016	3.2	202.9	14.8	5.9	999.0	86.5

^{a)} The medium was tenfold diluted nutrient broth containing about 11 mg/l of atrazine. Adjustment of pH was carried out using phosphate buffer for pH 8 and citrate-phosphate buffer for pH 5. The results were shown as the mean of duplicated experiment.

^{b)} ATZ, atrazine; DEA, deethylatrazine; DIA, deisopropylatrazine.

atrazine degradation by the bacteria at stationary phase.

4. Production of *N*-Dealkylated Metabolites from Atrazine

The isolates RK016 and RK014 were cultured for 3 months in 1/10NB containing about 11 mg/l of atrazine at pH 5 and pH 8. As shown in Table 2, deethylatrazine (DEA) and deisopropylatrazine (DIA) were detected. In the control cultures, DEA and DIA were detected at the same level of original amount. Significantly higher amounts of DEA and DIA in the cultures of RK014 and RK016 compared to the control cultures suggest that these metabolites were produced by the bacteria as metabolites. The amount of DEA was much higher than DIA. These results were supported by previous reports that the microbial degradation of atrazine started from the de-ethylation or the deisopropylation of the side chains.^{2-6,11)} Only DEA has been detected with atrazine in the environment.¹²⁻¹⁴⁾ It is possible that the isolates in this study play a major role in the microbial degradation of atrazine in soil.

At pH 5, the decrease of atrazine in the control culture indicated the chemical degradation under acidic conditions. Almost no accumulation of DEA and DIA was observed. This agreed with the findings that the chemical degradation of chloro-*s*-triazines under acidic conditions occurs through hydrolysis of chlorine.¹⁵⁾

At pH 8, the decrease in mass balance in both bacterial cultures compared with that in

control indicated the further degradation of atrazine metabolites by bacteria.

5. Relation with Slow Microbial Degradation in Soil

The degradation activity was highest at pH 8. The accumulation of DEA was observed in the bacterial degradation. These results suggested that the bacterial isolates were involved in the microbial degradation of atrazine in soil. It was found that atrazine was degraded by both bacteria RK014 and RK016 at stationary phase. This can be one of the reasons why microbial degradation of atrazine in soil was very slow in spite of the presence of degrading microorganisms. The insufficient cell density of the degrading microorganisms in soil also can be one of the reasons for the slow microbial degradation in soil. The cell density higher than 10⁸ or 10⁹ cfu/ml, which resulted in the high rate of atrazine degradation in the cultures, has not been observed in soil, where the population density of degraders was 10⁵ to 10⁶ MPN/g-soil.¹⁾ It is still unknown what mechanisms are involved in this atrazine transformation by these bacteria at the stationary phase. Further experiment on atrazine-degrading enzymes involved and their function at low and high pH should be conducted.

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要 約

定常期にある土壌細菌によるアトラジンの分解

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アトラジン分解細菌 RK014 株と RK016 株がそれぞれ安城土壌(日本)および Pak Chong 土壌(タイ)から単離された。RK014 株はグラム陰性の胞子を形成しない非発酵性かん菌であった。RK016 株は *Bacillus* 属細菌と同定された。10 分の 1 希釈の普通ブイヨン培地中で、定常期にある細菌によりアトラジンは分解された。急速な分解には、 $10^8 \sim 10^9$ cfu/ml 以上の高い初期菌密度が必要であった。pH 条件が 5 よりも 8 のほうが分解速度が高かった。Deethylatrazine が両菌株の主要代謝産物であった。このようなアトラジン分解細菌の性質が、広く土壌中に 10^5 MPN/g 土壌で分解菌が存在するにもかかわらず、土壌中でのアトラジンの微生物分解が非常に遅い原因となっていることが示唆された。